**Response to Reviews**

JoVE57464

***Editorial comments***  
*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.  
  
2. Please use SI units, e.g. please use “µL” instead of “µl”. Please leave a white space between the values and the units.  
  
3. Please define all abbreviations before use.  
  
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.  
  
5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.  
  
6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.  
  
7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.  
  
8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.  
  
9. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.  
  
10. Please leave a blank line between all protocol steps as well as Notes.  
  
11. Protocol: 1: How to prepare the soil? In which container? Using which instrument? Please clearly describe all steps.  
  
12. Protocol: 1.1: Sieving how? Using what? Which equipment? Which container?  
  
13. Protocol: 2.1: How exactly is the pH adjusted? How much of each component is used? How they are mixed? Please describe all the step clearly. Please only mention the actions. Please move the discussions to the Discussion section.  
  
14. Protocol: 2.2: “Combine” how? In which container?  
  
15. Protocol: 2.4: Centrifuge at which temperature? Room temperature? Please use “x g” for the centrifuge force. “Pour off” how? With what?  
  
16. Protocol: 2.4: Note 1: How much longer approximately? How to determine the time?  
  
17. Protocol: 2.4: Notes 2-4 can be moved to Discussion.  
  
18. Protocol: 2.5: Repeat what? 2.4? or 2.3 and 2.4?  
  
19. Protocol: 2.6: Please describe the step clearly.  
  
20. Protocol: 2.7: Please define ddH2O first. How is that done? How much water? Air-dry for how long? Or oven-dry for how long?  
  
21. Protocol: 2.8: How is the quantification done? How is the Analyzer used? Please clearly describe the steps or refer to appropriate references or protocols.  
  
22. Protocol: 3.1: “Place” using what?  
  
23. Protocol:*[*4.1.1.1*](https://webmail.illinois.edu/owa/redir.aspx?C=v_J_3OCyDJ_yBTLAaFZO0lvZ_XaII6lQnn3v4bH7npN6gB13-DXVCA..&URL=http%3a%2f%2f4.1.1.1)*: Please use the imperative tense. Please move the discussion to the Discussion section.  
  
24. Protocol:*[*4.2.2.2*](https://webmail.illinois.edu/owa/redir.aspx?C=0J2J3wnfSGFJoKFbI1uRUvtI1jRSgaxBX7eFkVla1WV6gB13-DXVCA..&URL=http%3a%2f%2f4.2.2.2)*: Please clearly describe the steps. Please move the discussion to the Discussion.  
  
25. Protocol:*[*4.1.1.3*](https://webmail.illinois.edu/owa/redir.aspx?C=qHC6eza212Pf5iq1xXzfMFgr1959RlXEOOPXd_q_9H96gB13-DXVCA..&URL=http%3a%2f%2f4.1.1.3)*: Please describe the steps clearly using the imperative tense. “Collect replicate spectra” how? Please move the discussion to the Discussion.  
  
26. Protocol: 4.1.2, 4.1.3, 4.2.1, 4.2.2, 4.3, 4.4: Please describe the steps clearly using the imperative tense. Please include all the actions. Please ensure you answer the “how” question, i.e., how is the step performed? Please move the discussion to the Discussion.  
  
27. Protocol: For steps that involve software or analyzing devices, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we’re looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.  
  
28. Please avoid using sub-sections in the “Representative Results” and “Discussion”.  
  
29. Figure 1: Please add the unit to each axis.*This change has been made.

*30. Figures 2, 4, 5, 6: Please describe the shadow regions.*This change has been made in the figure captions.

*31. Figure 6: Please leave a white space between the value and the unit, i.e., “500 °C” instead of “500°C”.*This change has been made.

*32. If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] et al.[REFERENCE]”.  
  
33. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.*  
  
*34. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*  
This change has been made.

***Reviewers' comments:******Reviewer #1:***  
*Manuscript Summary:  
This manuscript describes spectral subtraction method for improving soil organic matter composition analysis by FTIR spectroscopy.  
Overall it is a well put together manuscript which provides the step to step procedure of spectral subtraction method for SOM characterization by FTIR analysis and its interpretation. There is one general area which should be clarified before the paper is published.  
  
-NaOCl oxidation can remove relatively labile OM leaving stable OM and minerals intact in the soil sample. This incident might also be reflected in the FTIR spectra which possibly could introduce terrible artifacts in the subtracted SOM spectra (Yeasmin et al., 2017 Geoderma). Anyone should take this phenomenon into consideration during interpreting the SOM spectra which are subtracted by NaOCl oxidized sample spectra. On the other hand, in some cases aliphatic carbon groups which are generally expected to be oxidized can be intact after NaOCl treatment (Yeasmin et al., 2017 GCA). This could be due to the presence of large amounts of fresh plant materials containing substantial amounts of oxidation resistant fatty acids, waxes (aliphatics) compounds (Von Lu¨tzow et al., 2007). Thus, its quite difficult to use this NaOCl oxidation procedure for this spectral subtraction purpose without knowing the sample itself. The author has already mentioned some this fact very briefly. I think this fact need little bit more clearance in the manuscript.*

We appreciate the constructive feedback. We agree that more detail could be added on this issue, and have accordingly incorporated the following at L283-298:

“Chemical oxidations can be an attractive alternative to ashing for supplying mineral backgrounds because they generally preserve mineral structure and thus avoid artifactual absorbance features in reference spectra and the resulting subtraction spectrum14,22. For example, Soil A lost 89% of SOC by NaOCl oxidation compared to 97% by ashing (Table 2) while preserving mineral absorbance features altered by ashing (Fig. 2). On the other hand, incomplete and potentially selective removal of SOM means that NaOCl-resistant OM will not be characterized5,36,37, requiring careful interpretation of the subtraction spectrum14.”

An additional point has been added the beginning of the Discussion at L258-268:

“The method of removing SOM carries two considerations: 1) the amount of SOM removed, and 2) absorbance artifacts in the resulting mineral reference spectrum. It is fortunately possible— and arguably necessary— to identify and quantity these issues in order to avoid biased interpretations on SOM composition from the subtraction spectrum. Ideally, spectral subtractions would employ a mineral-only reference spectrum to yield a spectrum of SOM. In reality, the resulting subtraction spectrum exhibits absorbances corresponding to SOM that are enhanced relative to the original soil spectrum. This is because non-linear absorbance of mineral components in soil samples prevents complete subtraction of all mineral absorbances. Additionally, artifactual mineral absorbance and/or in incomplete removal of SOM (e.g., NaOCl oxidation) and thus its enhancement in subtraction spectra limit interpretations of specific absorbance features or interpretation of the total SOM pool.”

***Reviewer #2:*** *Manuscript Summary:  
This manuscript discusses a method for providing enhanced soil organic matter spectra in the presence of major soil minerals. This has been an interesting topic for many years and this paper continues the discussion.  
  
Major Concerns:  
The first issue is to do with enhancing the organic matter signal by oxidation. The paper discusses this aspect clearly and fairly thoroughly except that it fails to consider some alternative oxidizing agents and lower temperature oxidation. The second issue is to do with spectral subtraction. This is also well covered except that it does not consider or try to deal with the non-linear spectral response of the DRIFT method.*

The Introduction and Discussion address drawbacks of the general methods of SOM removal (thermal combustion and wet chemical oxidation) as well as the specific examples of these two removal methods. To further emphasize the issues with this removal methods, we have made following changes have been made to the end of the Introduction (L95-109):

“The two example methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion (‘ashing’) and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl) ~~as an example oxidant~~. It should be noted that these are examples of commonly SOM removal methods, rather than prescriptive recommendations. Given artifacts of combustion and oxidation methods are increasingly quantified and alternative SOM removal methods are evaluated (e.g., low-temperature ashing)14. High-temperature ashing was one of the first methods used to obtain mineral-enriched reference spectra for performing subtractions, initially for OM-enriched samples derived from soils (e.g., dissolved organic matter, litter)15,16 followed by application to soils17,18. The ~~use of~~demonstrated chemical oxidation to remove SOM is based on the method of NaOCl oxidation described by Anderson19. This was originally developed for minimizing OM content of soils prior to X-ray diffraction (XRD) analyses, and has been investigated as a potential chemical fractionation sensitive to SOM stabilization20,21. Both high-temperature removal and chemical oxidation using NaOCl can entail soil-specific artifacts and have limitations on spectral interpretation that should be considered when selecting a method of SOM removal14,22.”

Additional changes to address this issue in the Introduction and Discussion sections have made in response to specific reviewer comments (please see below).

*Minor Concerns:  
Protocols:  
Part 3.2. High temperature oxidation. Why was 550C chosen? It is well known that clay minerals such as kaolinite decompose at this temperature. Lower temperature for prolonged times (~350C for overnight heating) mostly avoids mineralogical changes. The longer ignition time helps remove most of the OM even though 350C is below the ignition temperature of soil OM.*

We discuss the selection of 550 °C in the Introduction at L95-100:

“The two example methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion (‘ashing’) and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl) ~~as an example oxidant~~. Differences in High-temperature ashing was one of the first methods used to obtain mineral-enriched reference spectra for performing subtractions, initially for OM-enriched samples derived from soils (e.g., dissolved organic matter, litter)1,2 followed by application to soils3,4.”

As indicated in the Introduction, 550 °C is an example of a potential method of SOM removal, rather than a prescriptive recommendation. Issues with these example SOM removal methods are reviewed and alternatives discussed in greater detail in the Introduction and Discussion sections.

To further clarify that the two SOM removal methods are meant to be examples, the following changes have been made to the end of the Introduction (L95-109):

“The two example methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion (‘ashing’) and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl) ~~as an example oxidant~~. It should be stressed that these are examples of commonly SOM removal methods, rather than prescriptive recommendations. Given artifacts of combustion and oxidation methods are increasingly quantified and alternative SOM removal methods are evaluated (e.g., low-temperature ashing)14. High-temperature ashing was one of the first methods used to obtain mineral-enriched reference spectra for performing subtractions, initially for OM-enriched samples derived from soils (e.g., dissolved organic matter, litter)15,16 followed by application to soils17,18. The ~~use of~~demonstrated chemical oxidation to remove SOM is based on the method of NaOCl oxidation described by Anderson19. This was originally developed for minimizing OM content of soils prior to X-ray diffraction (XRD) analyses, and has been investigated as a potential chemical fractionation sensitive to SOM stabilization20,21. Both high-temperature removal and chemical oxidation using NaOCl can entail soil-specific artifacts and have limitations on spectral interpretation that should be considered when selecting a method of SOM removal14,22.”

*You don't say anything about checking first the mineralogy of the sample to be heated. Some samples cannot be subjected to heating because they decompose even at low temperatures (~120C) such as AlOOH and FeOOH minerals.*

The important issue of mineral artifacts due to thermal combustion for SOM removal is addressed at L466-475.

“Knowledge of a soil sample’s mineralogy can also be used *a priori* to determine a maximum ashing temperature that avoids mineral alteration (e.g., interlayer collapse of kaolinite at 350 °C). Mineral artifacts can also be identified and anticipated by subjecting a soil sample to ashing at varying temperatures and comparing the resulting spectra with the spectra of untreated soil and pure mineral5,6. One may also constrain sample set mineralogy in order to maintain subtraction artifacts of ashing constant across samples. Finally, lower ashing temperatures (e.g., 350 °C) can be used to enhance some organic bands5,7. Similar to chemical oxidations, the use of lower temperatures generally comes at a cost of lower SOM removal8, as evidenced for the example soils (Table 2) and may engender selective loss of functional groups5.”

This discussion notes the potential of low-temperature ashing to also incur mineral (and organic) artifacts.

We agree that low-temperature ashing is a less artifact-prone method of SOM removal. This important point has been further stressed at L537-538:

“Alternative SOM removal methods have yet to be systematically investigated. Promising removal methods include low-temperature ashing (often performed under low pressure) and for chemical oxidations, hydrogen peroxide and sodium peroxodisulphate21.”

Additionally, the potential of low-temperature ashing as an alternative SOM removal method has been added to the Introduction (L97-100):

“The two ~~example~~ methods of SOM removal for obtaining a mineral-enriched reference spectrum are (1) high-temperature combustion (‘ashing’) and (2) chemical oxidation, using dilute sodium hypochlorite (NaOCl)~~as an example oxidant.~~ It should be stressed that these are examples of commonly SOM removal methods, rather than prescriptive recommendations. Other methods of SOM removal methods may offer reduced mineral artifacts and/or enhanced removal rates (e.g., low-temperature ashing) 14.

*L178: "grinding beyond 2000 μm does not necessarily improve spectral quality for chemometric predictions". I dispute this. Published work shows an improvement of about 0.1 R-square units for prediction modelling by fine grinding to <0.1 mm.*

The statement in question is a summary of a particular study that finds no significant benefit to grinding beyond this particle size— at least for specific soils and experimental objectives of these studies. We sought to be cautious and did not recommend against grinding beyond a maximum particle size of 2000 μm. For example, we write that grinding does not “necessarily” improve spectral quality. Our intention was to connote that for some soils and/or experimental objectives it may not be a beneficial soil pre-treatment.

To clarify this, we have modified the section at l79-189 as follows:

“Note: Though a maximum particle size of 250 – 100 µm has been considered ideal because the aperture of most FTIR spectrometers is 1000 µm 26, recent evaluations suggest that grinding beyond 2000 µm does not necessarily improve spectral quality for chemometric predictions 27,28. ~~However,~~ More finely ground soil samples tend to yield~~ed~~ enhanced (sharper) absorbance peaks and exhibited decreased absorbance variability 27, which would be expected to benefit spectral subtractions. On the other hand, some studies have found that grinding (e.g., to 200 µm) can induce more heterogeneity in particle size distribution than standard sieving (e.g., 2000 µm)29,30. The effect of this on spectral quality is likely to depend on experiment objectives (e.g., interpretation of absorbance bands, chemometric prediction of soil C) and soil types29. It is therefore recommended that grinding size be assessed for particular set of soils and objectives.”

*L203: These days, mirror type of background referenced are used, such as stainless-steel, gold or SiC. These reduce the problems of contamination and humidity effects on KBr.*

We have added this clarification at L214-217:

“For example, collection of spectra under ambient atmosphere may entail small fluctuations in humidity and CO2 that can cause changes in absorbance spectra. Newer spectrometers may have mirrors (e.g., gold, SiC) that can potentially reduce humidity effects.”

*Part 4. You do not discuss linearizing the DRIFT spectral responses by applying a non-linear transform such as the Kubelka Munk or a power function. This may help balance the relative different subtraction factors for OM and mineralogy.*

We thank the reviewer for this suggestion; it has been added as a possibility in the Discussion section at L327-329:

“It is important to note that the SF suggested by software is also influenced by the wavenumber range selected for the subtraction because the software optimizes a subtraction for the defined spectral region. Additional benefits of softwares include spectral processing transformations (e.g., Kubelka-Munk, power functions) to improve spectral quality (e.g., linear baselines) prior to performing subtractions.”

*L266: SOM removal. I guess that the different SOM oxidation by chemical versus ignition is similar to the comparison between the dichromate oxidation with Walkely-Black and Leco ignition. In the chemical method, some organic species such as soil char material is not easily chemically oxidized.*

The Discussion describes incomplete removal of SOM by NaOCl oxidation (or other removal methods, including low-temperature ashing) as a key trade-off of this method. This has been further clarified at L283-289:

“Chemical oxidations can be an attractive alternative to ashing for supplying mineral backgrounds because they generally preserve mineral structure and thus avoid artifactual absorbance features in reference spectra and the resulting subtraction spectrum14,22. For example, Soil A lost 89% of SOC by NaOCl oxidation compared to 97% by ashing (Table 2) while preserving mineral absorbance features altered by ashing (Fig. 2). On the other hand, incomplete and potentially selective removal of SOM means that NaOCl-resistant OM will not be characterized5,36,37, requiring careful interpretation of the subtraction spectrum14.”

Additionally, the opening Discussion paragraph has been modified to underscore this limitation of spectral subtractions, at L258-268:

“The method of removing SOM carries two considerations: 1) the amount of SOM removed, and 2) absorbance artifacts in the resulting mineral reference spectrum. It is fortunately possible— and arguably necessary— to identify and quantity these issues in order to avoid biased interpretations on SOM composition from the subtraction spectrum. Ideally, spectral subtractions would employ a mineral-only reference spectrum to yield a spectrum of SOM. In reality, the resulting subtraction spectrum exhibits absorbances corresponding to SOM that are enhanced relative to the original soil spectrum. This is because non-linear absorbance of mineral components in soil samples prevents complete subtraction of all mineral absorbances. Additionally, artifactual mineral absorbance and/or in incomplete removal of SOM (e.g., NaOCl oxidation) and thus its enhancement in subtraction spectra limit interpretations of specific absorbance features or interpretation of the total SOM pool.”

*You need to stress the importance of detailed knowledge of the interpretation of MIR soil spectra. Failure in this regard may easily lead to inappropriate subtraction and thus complete miss-interpretation.*

We have clarified the overall limitation of spectral subtractions (semi-quantitative, operational) and need for careful interpretation of absorbances to infer relative changes in organic functional group composition of SOM, at L374-377:

“It is essential that subtraction spectra be understood as an operational method to assess relative changes in absorbances that can be assigned to organic functional groups with varying certainly depending on sample type and absorbance range (due to potential overlapping bands)4.”

**References**

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10 Nduwamungu, C., Ziadi, N., Tremblay, G. F. & Parent, L.-É. Near-Infrared Reflectance Spectroscopy Prediction of Soil Properties: Effects of Sample Cups and Preparation All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher. *Soil Science Society of America Journal.* **73** (6), 1896-1903, doi:10.2136/sssaj2008.0213, (2009).